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14. ABSTRACT

The purpose of this award is to test in preclinical models the hypothesis that cytotoxic conjugates of elemental selenium and proteins are safe and effective for the systemic therapy of invasive breast cancer. The grant has three specific aims, 1) to evaluate the safety and efficacy of systemically administered Se(0)-protein conjugates in athymic nude mice bearing xenografts of human breast cancer cells, 2) to assess the functional integrity of conjugate-treated normal human hematopoietic stem cells, and 3) to determine by use of the combination index method how Se(0)-protein conjugates interact with standard chemotherapeutic agents that are commonly used in the treatment of breast cancer. We report here on the development and evaluation of improved methods for the generation of high-potency cytotoxic Se(0)-protein conjugates, the interactions of Se(0)-protein conjugates with standard chemotherapeutic agents, and the interference of certain antibiotics with the photochemical generation of Se(0)-protein conjugates.

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Introduction

Early detection, adjuvant hormone therapy, and adjuvant chemotherapy have improved survival rates in breast cancer. However, for most patients with advanced disease, the prognosis remains poor. In the 1990s, large numbers of breast cancer patients were treated with high-dose chemotherapy and autologous hematopoietic stem cell transplants. The expectation was that the dose escalation afforded by the hematopoietic stem cell transplants would have a major impact on survival. However, controlled trials have failed to show a significant advantage of high-dose chemotherapy over standard therapy. It thus appears that currently available forms of chemotherapy - even when used at very high doses - are rarely able to eradicate the disease in patients with high-risk breast cancer.

Most of the currently used anti-cancer drugs were developed based on their good performance in leukemia/lymphoma-based screening systems. Not surprisingly, they tend to perform best when used in the treatment of leukemias and lymphomas. Major breakthroughs in the treatment of breast cancer will most likely require new agents whose mechanism of action is different from that of conventional anti-cancer drugs.

Grant application W81XWH-04-1-0525 proposed to assess the safety and efficacy of a novel class of cytotoxic agents whose mechanism of action appears to be very different from that of established anti-cancer drugs. The novel cytotoxic agents consist of high-affinity conjugates of extremely small particles of elemental selenium and proteins or lipoproteins. The (lipo)protein component acts as a Trojan horse that delivers the cytotoxic entity (selenium in oxidation state zero) to breast cancer cells as part of a physiological process. It exploits the fact that breast cancer cells have an increased requirement for serum albumin (and possibly lipoproteins) and, therefore, an increased capacity to bind and internalize albumin by an endocytotic process. Once inside their target cells, Se(0)-protein conjugates act as air oxidation catalysts that rapidly deplete cells of glutathione, induce a loss of mitochondrial potential and plasma membrane asymmetry, and activate caspases. The experience with tumor cell lines suggests that the cytotoxic action of Se(0)-protein conjugates is not cell-cycle specific and is not affected by most drug resistance mechanisms.

Incorporating cytotoxic Se(0)-albumin conjugates into the treatment of invasive breast cancer may prove particularly rewarding because breast cancer tissue is known to accumulate exceptionally large quantities of serum albumin. Typically, about one fifth of the cytosolic protein content of breast cancer cells consists of serum albumin. Despite low blood flow in breast cancer tissue, albumin clearance is very high in breast cancer tissue, and albumin extraction is 3-20 times higher than in any normal tissue. The inverse correlation between albumin content and estrogen-receptor expression suggests that Se(0)-protein conjugates may prove particularly useful in the treatment of estrogen-receptor negative breast cancer.

Grant application W81XWH-04-1-0525 was designed to test the hypothesis that proteinated subnano particles of elemental selenium can be developed into safe and effective agents for the treatment of invasive breast cancer. The proposal had 3 specific aims, 1) to evaluate the safety and efficacy of systemically administered Se(0)-protein conjugates in athymic nude mice bearing xenografts of MCF7 or MDA-MB-435 human breast cancer cells, 2) to assess the functional integrity of conjugate-treated normal

human hematopoietic stem cells, and 3) to determine by use of the combination index method how Se(0)-protein conjugates interact with standard chemotherapeutic agents that are commonly used in the treatment of invasive breast cancer.

Body

Task 1: Preparation and In Vivo Evaluation of High-Potency Cytotoxic Se(0)-protein Conjugates.

The initial in vitro evaluation of cytotoxic Se(0)-protein conjugates under high serum conditions showed that it would be very difficult - if not impossible - to prepare sufficiently potent Se(0)-protein conjugates using the selone dye MC54 as a starting material. The main problem was that as soon as dye concentrations reached about 80 μM, the photobleaching process slowed down dramatically. We attributed the problem to the lipophilic nature of MC54 and its tendency to form aggregates in aqueous media. Two potential remedies were explored. 1) We had two analogues of MC54 synthesized that had less lipophilic benzoxazole or benzthiazole back rings instead of the naphth[2,1-d]thiazole back ring of MC54. 2) We generated Se(0)-protein conjugates by the chemical reduction of selenium dioxide in the presence of protein.

Both approaches were partially successful. For example, both dye analogues generated fluorescent and cytotoxic photoproducts at the expected rate, were more soluble in water than MC54, and displayed spectral characteristics indicative of their improved solubility in water. However, the gain in water solubility was too small to make the preparation of truly high-potency conjugates feasible.

Initial attempts to generate Se(0)-protein conjugates by the reduction of selenium dioxide with ascorbic acid or glutathione yielded preparations with significant cytotoxic activity (e.g. 4-log depletions of tumor cells within 1 hour), but cytotoxic potencies fell almost two orders of magnitude short of those achieved by the photobleaching of selone dyes. A majority of Se(0) particles generated under these conditions were simply too large to be biologically active. Furthermore, Se(0)-albumin conjugates generated by the chemical reduction of selenium dioxide quickly became unstable, and macroscopic precipitates of Se(0) and albumin formed within a matter of days. Precipitate formation was accompanied by a drop of pH from 7.2 to approximately 5.2. While the pH shift readily explains the precipitation of albumin, the underlying chemistry that leads to the pH shift is not yet understood.

Attempts to use cyclodextrins to control particle size were not successful, as the cyclodextrins did not exchange Se(0) with albumin. However, we recently obtained very encouraging results with a series of selenite esters that may represent a major technical breakthrough. The esters were developed by our long-time collaborator W.H.H. Gunther to mimic the slow release of Se by selone photosensitizers during photobleaching, speculating that this "one-atom-at-a-time" release of Se may be crucial for the formation of very small (and therefore cytotoxic) particles of Se(0). Se(0)-albumin conjugates prepared from this new starting material are stable for at least 2 months (long-term monitoring still in progress), essentially free of large aggregates, and very active against breast cancer cells. They can be prepared in large quantities at a fraction of the costs of

photochemically generated Se(0)-protein conjugates. A surprise finding is that with Se(0)-protein conjugates derived from Gunther's selenite esters we are no longer limited to Se:protein loading ratios of about 6:1 or less, and that loading ratios in excess of 6:1 result improve cytotoxic activity. The highest loading ratio tested so far is 66:1. Conjugates with loading ratios as high as 600:1 have been synthesized and appear to be stable but have not yet been tested for cytotoxic activity. Selected samples are currently being analyzed by mass spectroscopy at M-Scan (West Chester, PA) to determine if our calculated loading ratios are indeed correct. Optimal loading ratios will be determined during the 1-year no cost extension of the grant.

In summary, the preparation of high-potency Se(0)-protein conjugates has required more effort than I had anticipated when I wrote the original application. Making this effort undoubtedly slowed down progress on other specific aims. However, the technical breakthrough that seems to have been achieved eventually may turn out to be critical for the long-term objective of this project.

Interference of Antibiotics With Generation of Cytotoxic Se(0)-protein Conjugates and Green-Fluorescent Photoproduct-Albumin Conjugates

During the second year of support, a leak in a RO water storage tank caused extensive flooding in our laboratory area. After the flooding incidence, we had major problems with infections, which forced us to add antibiotics (penicillin/streptomycin) to our culture media. Shortly after the flooding incidence, a failure in the regulatory circuit of the air conditioning system maintained room temperatures in the laboratory at 113 °F for an extended period of time. This caused multiple equipment failures and a loss of all cell cultures. When we reestablished the lost cultures from frozen stock, previously sensitive tumor cell lines appeared to have become resistant to freshly prepared cytotoxic conjugates. The finding was confirmed with multiple cell lines from authentic stock, ruling out an accidental mix-up of cell lines. We initially suspected heat damage to equipment and/or reagents as the cause of the problem but eventually realized that antibiotics had been added not only to the culture medium used for cell maintenance and clonal assays but also to the medium used for the production of cytotoxic conjugates. As we found out, the presence of antibiotics greatly reduced the cytotoxic potency of photobleached MC54 and the production of green-fluorescent photoproduct-albumin conjugates. It did so at least in part by causing extensive dye aggregation. A similar suppression of fluorescent conjugate formation was achieved by the drug flufenamic acid, suggesting that access to the Type II binding site of albumin was critical.

Studies on the interference of antibiotics with conjugate formation were not part of the original research plan. However, we decided to investigate the problem for two reasons, 1) because being able to generate cytotoxic conjugates of predictable potency was essential for all three specific aims of the grant, and 2) because of its potential implications for potential clinical applications of Se(0)-protein conjugates. Cancer patients are often neutropenic and, therefore, at increased risk for infections. Prophylaxis with antibiotics reduces the risk of infections, but, as this study showed, could potentially interfere with the cytotoxic activity of Se(0)-protein conjugates if autologous serum is used for the preparation of conjugates. As our experience shows, spectroscopic

monitoring of the photobleaching process provides a quick and simple way to detect problematic interactions with antibiotics.

Task 3: Interactions of Cytotoxic Se(0)-protein Conjugates With Other Chemotherapeutic Agents

The quantitative analysis by the combination index method of the interactions of cytotoxic Se(0)-protein conjugates with other chemotherapeutic agents progressed well during the third year of funding and will be completed during the 1-year no-cost extension. The interim results can be summarized as follows.

Se(0) protein conjugates plus doxorubicin: Consistently strong synergism (combination index: 0.1 - 0.3) at all combinations when breast cancer cells were exposed to both agents simultaneously. When breast cancer cells were exposed sequentially to Se(0)-protein conjugates (first) and doxorubicin (second), combined cytotoxic effects ranged from nearly additive (combination index: 0.9 - 1.1) to slightly synergistic (combination index: 0.85 - 0.9) or synergistic (combination index: 0.3 - 0.7) depending on the particular drug combination used.

Se(0)-protein conjugates plus 4-hydroperoxycyclophosphamide: Simultaneous exposure showed a nearly additive (combination index: 0.9 - 1.1) cytotoxic effect for all combinations.

Se(0)-protein conjugates plus cisplatin: Simultaneous exposure showed antagonism (combination index: 1.45 - 3.3) for two combinations and a slightly antagonistic (combination index: 1.1 - 1.2) or nearly additive effect (combination index: 0.9 - 1.1) for the two remaining combinations. The latter result is a positive surprise, as early pilot experiments with leukemia cells had suggested that all combinations of cisplatin and Se(0)-protein conjugates would be highly antagonistic. It is conceivable that antagonism can be further reduced by exposing breast cancer cells *sequentially* to Se(0)-protein conjugates first and cisplatin second.

One-Year No Cost Extension

Since work on this project was delayed by a time-consuming move of my laboratory to a different building, I have requested and received a 1-year no-cost extension.

Key Research Accomplishments

- The in vitro analysis of cytotoxic Se(0)-protein conjugates under high-serum conditions has shown that it is very difficult to produce conjugates with sufficient potency using the selenomerocyanine dye MC54 as a starting material. We have developed several potential remedies for this problem. The most promising of them is a novel method of generating Se(0)-protein conjugates by a chemical reduction of Se(IV).

- We made the unexpected observation that antibiotics (penicillin/streptomycin) interfere with the generation of cytotoxic and fluorescent conjugates if they are present

during the photobleaching of the selone dye. The finding has practical implications for future clinical applications of Se(0)-protein conjugates.

- The quantitative evaluation (using the combination index method of Chou & Talalay) of the cytotoxic effect of Se(0)-protein conjugates used in combination with other chemotherapeutic agents is progressing well with combinations of Se(0)-protein conjugates and doxorubicin showing strong synergism, combinations of Se(0)-protein conjugates and 4-hydroperoxycyclophosphamide showing mostly additive effects, and combinations Se(0)-protein conjugates and cisplatin showing less antagonism than anticipated.

Reportable Outcomes

***New Funding**:*

While X81XWWH-04-1-0525 was in progress, I applied for and received a 16-month pilot grant entitled "Nano-Selenium for the Mitigation of Radiation Injuries" (U19-AI067734) from the Center for Medical Countermeasures Against Radiological Terrorism (CMCRT). Although the CMCRT pilot grant focused on selenium compounds and relatively large particles elemental selenium with anti-oxidant rather than pro-oxidant properties, the grant clearly benefited from experience we had gained during our work on W81XWH-04-1-0525. The most exciting result of the CMCRT pilot grant is that nutritional supplementation with high-dose selenium initiated *after* radiation exposure prevented otherwise fatal radiation damage to kidneys and mitigates - to a lesser extent - radiation damage to heart, lungs, and liver. Dietary supplementation was well tolerated with no animals showing signs of selenium toxicity. Based on mechanistic consideration, it is reasonable to speculate that the same high-dose selenium regimen might also mitigate organ damage caused by certain chemotherapeutic agents. Therefore, data generated by the CMCRT pilot grant may also have potentially important implications for cancer patients (including breast cancer patients) who sustain damage to normal tissue as a consequence of radiation therapy or chemotherapy.

Conclusions

The limited solubility of the pro-drug MC54 makes the production of high-potency Se(0)-protein impractical. We have developed and evaluated several potential remedies for this problem. The remedies are based on the use of less lipophilic selone photosensitizers or chemical rather than photochemical) methods of generating elemental selenium. The most promising results were obtained with the chemical reduction of slow-release selenite esters in the presence of serum albumin. The resulting Se(0)-albumin conjugates are highly cytotoxic, have along shelf life, cover a wide range of Se:protein loading ratios, and can be produced at a fraction of the costs of photochemically generated Se(0)-protein conjugates.

We have made the unexpected observation that therapeutic concentrations of antibiotics (penicillin/streptomycin) can interfere with the production of cytotoxic Se(0)-protein conjugates and green-fluorescent photoproduct-albumin conjugates. The observation has practical implications for future clinical applications of cytotoxic Se(0)-protein conjugates. The spectrophotometric analysis that was used to diagnose the problem provides a simple and rapid method to screen antibiotics for potential interference with cytotoxic conjugate formation.

Using Se(0)-protein conjugates in combination with established chemotherapeutic agents is feasible. A quantitative evaluation by the combination index method of Chou & Talalay has shown strong synergism with doxorubicin, mostly additive effects with 4-hydroperoxycyclophosphamide, and weaker than expected antagonism with cisplatin.

References

None

Appendix

None